

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
11 March 2004 (11.03.2004)

PCT

(10) International Publication Number  
**WO 2004/020406 A2**

(51) International Patent Classification<sup>7</sup>: **C07D**

(21) International Application Number:  
PCT/US2003/026968

(22) International Filing Date: 29 August 2003 (29.08.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/406,917 30 August 2002 (30.08.2002) US

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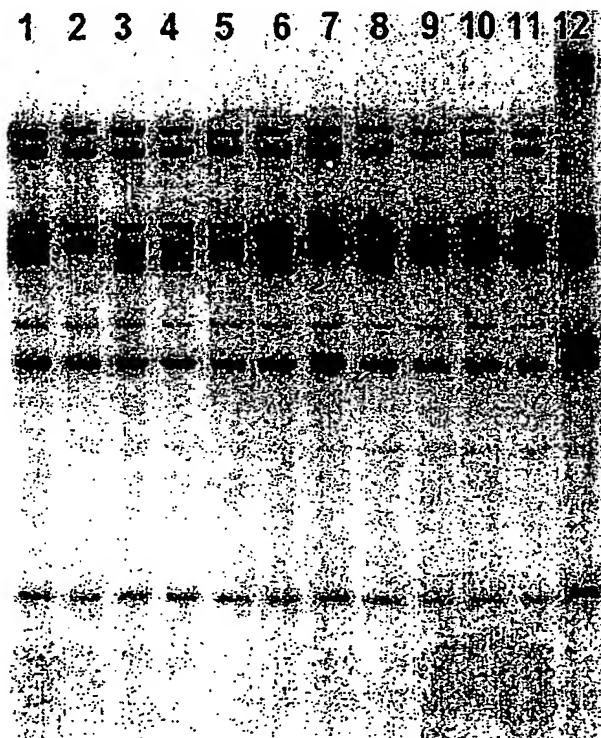
(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,

[Continued on next page]

(54) Title: **VARIANTS OF VIBRIO CHOLERAЕ O1 BIOTYPE E1 TOR WITH ATTRIBUTES OF CLASSICAL BIOTYPE**

(57) Abstract: The invention relates to novel types of *Vibrio cholerae* that are useful for vaccines and immunological compositions.



WO 2004/020406 A2

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ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,  
SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM,  
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**Published:**

— *without international search report and to be republished  
upon receipt of that report*

1/PRTS

**Variants of *Vibrio cholerae* O1 Biotype El Tor with Attributes of Classical Biotype**

5 BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to novel types of *Vibrio cholerae* that are useful for vaccines and immunological compositions.

10 2. Background Information

New epidemic strains of toxigenic *Vibrio cholerae* have appeared at least twice in recent human history (10). Strains of the classical biotype, which had probably been responsible for most of the epidemic disease in the 19<sup>th</sup> century and much of the 20<sup>th</sup> century, were largely replaced as the predominant cause of epidemic cholera by strains of the El Tor biotype in most of the regions where cholera is endemic, beginning in 1961. However, the classical biotype strains reemerged as a predominant epidemic strain in parts of Bangladesh in 1982 (8,25) and coexisted with the El Tor strains, causing disease until 1993. A second new epidemic strain, carrying the O139 rather than the O1 antigen, emerged in southern Asia in 1992 (7,24). The O139 and El Tor O1 strains continue to cause epidemics of cholera, and there are indications that the incidence of cholera due to the O139 serogroup is on the rise in parts of India and Bangladesh.

The classical and El Tor biotypes of *V. cholerae* are closely related in their O-antigen biosynthetic genes (21,31), although these two biotypes differ in many other regions of their genomes (2,16,17,29,30). Thus, O1 El Tor strains might have arisen following transfer of O1 antigen biosynthetic genes into a previously unknown environmental strain. Conversely, O139 and O1 El Tor strains are closely related in most parts of their genomes, but carry different O-antigen genes, suggesting the transfer of O139-specific genes from an unknown donor into a recipient El Tor strain (3,28). Similar conclusions about gene transfer have emerged from comparisons of

serogroups and sequences of diagnostic housekeeping genes of nonepidemic isolates (2).

#### SUMMARY OF THE INVENTION

5           The present inventors have identified a new variety of *V. cholerae* O1 that appears to be a hybrid of the classical and El Tor biotypes from hospitalized patients with acute diarrhea. The phenotypic strains that distinguish the classical and El Tor biotypes of *V. cholerae* O1 and important discriminating genotypic characteristics of the existence of such novel strains make them ideal for the development of new  
10 cholera vaccines.

          Three new types of *Vibrio cholerae* O1 (designated Matlab I, Matlab II, and Matlab III) have been isolated from cholera patients and characterized. These include 24 new strains, 2 of which are Matlab I, 1 of which is Matlab II, and 21 of which are Matlab III. Phenotypic traits characterized included serotype (Inaba, Ogawa), Voges  
15 Proskauer test, Polymyxin B sensitivity, chicken cell agglutination, and sensitivity to Group IV and Group V phages. Genotypic traits were analyzed using tcpA and ctxA PCR, and acfB and rstT probes. From their phenotypic traits, Matlab I, II and III appear to be hybrids of classical and El Tor biotypes.

          The invention provides isolated strains and biologically pure cultures of the  
20 Matlab I, II, and III, vaccines and pharmaceutical compositions containing them, and a method of immunization against *V. cholerae*.

          As used herein, a culture of *V. cholerae* is considered to be biologically pure if essentially all of the cholera organisms in the culture or products of the culture are from one strain or type. All colonies grown from the original culture should be  
25 identical to the original taking into account the possibility that a rare mutant strain might arise from the original strain. A mutant might theoretically be detected at a frequency of less than  $10^{-8}$  and these would not be detected when growing the strain using normal bacteriological procedures in which subcultures are prepared from the original.

Representative strains of Matlab I, II and III were deposited at the National Collection of Type Cultures, London, UK, on August 27, 2002 under accession nos. NC13269-01, NC13270-01 and NC13271-01.

Vaccines and pharmaceutical compositions of the invention can be prepared  
5 by any acceptable method. Formulation of cholera vaccines is familiar to those of skill in the art. In one embodiment, the vaccine contains heat- or formalin-killed whole cells selected from different biotypes and serotypes of Cholera in a total dose of  $10^{11}$  cells per dose. In a preferred embodiment, the vaccine includes previously known strains of cholera, including O139, as well as the strains of the invention.  
10 Optionally, the vaccine may include the cholera B subunit. The killed cells may be suspended in a pharmaceutically acceptable aqueous solution, including additional carriers, excipients and adjuvants, as will be known to persons of skill in the art. Techniques and formulations generally for use in pharmaceutical compositions and vaccines may be found in *Remington's Pharmaceutical Sciences*, Meade Publishing  
15 Co., Easton, Pa. One example of such a vaccine is DUKORAL®. Similar formulations can be made using the cholera strains of the present invention.

The vaccine may also be formulated into liposomes, as known in the art, for additional immunogenicity. Means for formulating liposomal compositions are described, *inter alia*, by Dima et al., Arch. Microbiol. Immunol. 60(1) 27-54 (2001);  
20 Harokopakis et al., Infect. Immun. 66(9):4299-304 (1998); Kalambaheti et al., Vaccine 16(2-3):201-7 (1998); Chaicumpa et al., Vaccine 16(7):678-84 (1998); Chaicumpa et al. J. Allergy Immunol. 8(2):87-94 (1990); Chaicumpa et al., Asian Pac. J. Allergy Immunol. 6(2):70-6 (1988).

In one preferred embodiment, the method of immunization against cholera  
25 comprises administering killed whole cells of the cholera strains of the invention in an effective amount to an individual in need of protection against cholera. Most preferably, the effective amount is contained in a single dose. Two or more doses may be necessary in some cases to establish a desired level of protection. The cells may be administered by any acceptable route, preferably oral. Preferably the cells are  
30 administered in the form of a vaccine or pharmaceutical composition, as described above.

In another preferred embodiment, the method of immunization against cholera comprises administering attenuated live cells of the cholera strains of the invention in an effective amount to an individual in need of protection against cholera.

Preferably, the effective amount is contained in a single dose.

5       The invention also includes a combination vaccine effective for immunization against the cholera strains of the invention, other known cholera strains and additional infectious organisms such as *E. coli* and rotavirus.

In one particularly preferred embodiment, the invention provides an isolated strain or biologically pure culture of *V. cholerae* having the identifying characteristics  
10 of a strain selected from the group consisting of Matlab I, Matlab II and Matlab III. The identifying characteristics may be phenotypic traits and/or genotypic traits. Most preferred is an isolated *Vibrio cholerae* strain having the characteristics of Matlab I, II or III, deposited at the National Collection of Type Cultures, London, UK, on August 27, 2002 with the depository numbers of NC13269-01, NC13270-01 and  
15 NC13271-01, respectively.

In another particularly preferred embodiment, the invention provides a vaccine or pharmaceutical/immunological composition for protection against cholera comprising *V. cholerae* having the identifying characteristics of *V. cholerae* selected from the group consisting of Matlab I, Matlab II and Matlab III. The vaccine or  
20 composition preferably comprises killed whole cells. The cells may be killed by any method known in the vaccine arts, for example, by heat or formalin. Preferably the vaccine is an oral vaccine. In one preferred embodiment, the number of organisms per dose of said *V. cholerae* is between about  $10^4$  and  $10^{16}$ . In another preferred embodiment, the strain of *V. cholerae* is combined with at least one additional strain  
25 of *V. cholerae*. The vaccine may also include a cholera toxoid. Also contemplated is a combination vaccine, which includes at least one component effective against an additional organism, such as rotavirus and enterotoxigenic *E. coli*. The vaccine/composition optionally includes a pharmaceutically acceptable excipient, adjuvant or carrier, preferably suitable for oral administration, such as a sterile saline  
30 buffered from about pH 7.1 to about pH 7.3.

In another particularly preferred embodiment, the invention includes a method of protecting humans against cholera comprising:

obtaining a *V. cholerae* culture comprising a *V. cholerae* having substantially all of the identifying characteristics of *V. cholerae* selected from the group consisting of Matlab I, Matlab II, and Matlab III; and

administering an effective amount of said culture to a human.

Preferably the culture is administered orally in a single dose.

Thus, the invention also includes the use of the strain of Matlab I, II, or III in a vaccine or immunological composition.

In yet another preferred embodiment, the invention includes an isolated strain of *V. cholerae* having the genotypic or genotypic characteristics of Matlab I, II, or III that has been attenuated, for example by excising the CTX prophage DNA that carries genes for cholera toxin. In this aspect, the invention includes such an isolated strain substantially does not secrete cholera toxin. Particularly preferred strains are those that are designated \_\_\_\_\_ deposited at the National Collection of Type Cultures, London, UK, on \_\_\_\_\_.

The invention further includes the use of all of the above-mentioned attenuated strains in a cholera vaccine or immunological/pharmaceutical composition. The vaccine or composition may be comprised of killed whole cells (killed, for example, by heat or formalin) or live cells, and is preferably an oral vaccine. The number of organisms per dose of said *V. cholerae* will generally be between about  $10^4$  and  $10^{16}$ . the vaccine or immunological composition may also include additional strains of *V. cholerae* and/or a cholera toxoid and may also be a combination vaccine that includes vaccine components effective against at least one organism in addition to *V. cholerae*. Particularly preferred for the combination vaccine are rotavirus and enterotoxigenic *E. coli*.

These and other aspects of the invention will be clear to those of skill in the art from the above description and the examples set forth below.

## BRIEF DESCRIPTION OF THE DRAWING

Figure 1 shows Bg/I restriction patterns of rRNA genes of *V. Cholerae* strains compared to those of selected typical strains of the El Tor and classical biotypes of *V. cholerae* O1. A Southern blot of Bg/I-digested genomic DNA was hybridized with the 7.5-kb BamHI fragment of *E. coli* rRNA clone pKK3535. Lanes (including strain designations and relevant characteristics): 1, toxigenic El Tor strain G-3669 (isolated in 1969 in Bangladesh; 2 through 10, strains MH-08 (Matlab type III), MG-116926 (Matlab type III), MG-117086 (Matlab type III), MG-116926 (Matlab type III), MG-116955 (Matlab type III), MG-116025 (Matlab type III), MG-116226 (Matlab type II), MJ-1485 (Matlab type I), and MJ-1236 (Matlab type I); 11, toxigenic El Tor strain 1849 (isolated in 2001); 12, toxigenic classical biotype strain (isolated in 1963 in Bangladesh).

## DETAILED DESCRIPTION OF THE INVENTION

### Materials and methods

Twenty four strains of *V. cholerae* isolated between 1991 and 1994 from hospitalized patients with acute diarrhea in the Matlab hospital, 45 km south of Dhaka, Bangladesh, were included in this study (34). The strains were isolated following standard methods of isolation of *V. cholerae* from stool samples which have been published in the WHO manual for isolation of enteric pathogens, and will be familiar to those of skill in the art. The basis of a retrospective examination of these strains was their unusual response to polymyxin B (50U), chicken cell agglutination (CCA), Voges-Proskauer (VP) reaction, and sensitivity to group IV and V phages, all of which are phenotypic traits commonly used to differentiate between the classical and El Tor biotypes. The 24 strains were reexamined for the above phenotypic characteristics by standard procedures.

The presence of the *ctxA* gene and the variants of the classical and El Tor *tcpA* genes were determined by a multiplex PCR assay (18). The expected size of the PCR amplicons was ascertained by electrophoresis in agarose gels. The identities of all PCT products were further verified with specific oligonucleotide probes. The probes for El Tor and classical biotype-specific CTX prophage repressor *rstR* were *SacI*-



*Xba*I fragments of pHK1 and pHK2, respectively (19). The *acfB* gene probe was prepared from the PCR amplicon with previously reported *acfB*-specific primers (13). The rRNA gene probe consisted of a 7.5-kb *Bam*HI fragment of *Escherichia coli* rRNA clone pKK3535 (5). Colony blots or Southern blots were prepared with nylon filters (Hybond; Amersham International plc., Aylesbury, UK) by standard methods (27). The probes were labeled by random priming (14) with a random-primer DNA labeling kit (Bethesda Research Laboratories, Gaithersburg, MD, USA) and [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol; Amersham). Colony blots and Southern blots were hybridized with the probes and autoradiographed as described by Faruque et al. (11-13).

#### EXAMPLE 1

We examined the commonly used phenotypic traits used to distinguish between the El Tor and classical biotypes of *V. cholerae* and differentiated the 24 strains into three types (Table 1), which we classified as Matlab types I, II, and III. Matlab type I included two strains belonging to the Inaba serotype that were resistant to both the El Tor-specific group IV and the classical biotype specific group V phages, negative by the CCA and VP tests (both are classical traits), and resistant to polymyxin B (an El Tor trait). Matlab type II included one strain belonging to the Ogawa serotype that was sensitive to the group IV phage but showed negative responses in the CCA and VP tests and was sensitive to polymyxin B, all of which are classical biotype characteristics. Matlab type III included 21 Ogawa strains that showed the sensitivity to phages and polymyxin B typical of the El Tor biotype but were negative by the CCA and VP tests (both classical biotype traits).

Table 1. Phenotypic traits of Matlab types I, II, and III of toxigenic *V. cholerae* O1 isolated from patients hospitalized with acute secretory diarrhea in Bangladesh

Type	No. of strains	No. of strains of serotype:	VP test <sup>a</sup>	Sensitivity to poly-myxin B (50U) <sup>b</sup>	CAA <sup>a</sup>	Phage sensitivity <sup>a</sup>	
						Group IV	Group V
						(El Tor biotype specific)(classical biotype specific)	
Matlab I	2	2	0	-	R	-	R
Matlab II	1	0	1	-	S	-	R
Matlab III	21	0	21	-	R	-	R
El Tor MAK757	1	0	1	+	R	+	R
Classical 154	1	0	1	-	S	-	S

## EXAMPLE 2

We also examined the genotypes of the strains. Genotypically, all of the strains carried the *ctxA* gene, a constituent gene of the CTX prophage that encodes cholera toxin (CT), and *acfB* and *tcpA*, which are located in different gene clusters (*acr* and *tcp* gene clusters) on the *V. cholerae* pathogenicity island. The type I strains appeared to belong more to the classical biotype because they carried the *tcpA* gene and the CTX prophage repressor gene (*rstR*) of the classical type (Table 2). The *tcpA* gene of the single type II strain was of the classical type, while the *rstR* gene was of the El Tor type. The six representative strains of *V. cholerae* representing Matlab III also carried the *tcpA* gene of the classical type. Five of the strains had the El Tor-type *rstR* gene, while one carried both the El Tor and classical *rstR* types.

Table 2

Strain	Matlab type	Yr of isolation	<i>tcpA</i> PCR	<i>ctxA</i> PCR	<i>acfB</i> (probe)	<i>rstR</i> (probe)
MJ-1236	I	1994	C	+	+	C
MJ-1485	I	1994	C	+	+	C
MG-116226	II	1991	C	+	+	E
MG-116025	III	1991	C	+	+	E
MG-116955	III	1991	C	+	+	E
MG-116926	III	1991	C	+	+	E,C
MG-117086	III	1991	C	+	+	E
MG-117159	III	1991	C	+	+	E
MH-08	III	1992	C	+	+	E
MAK757 (El Tor)	Ref	1937	E	+	+	E
154 (classical)	Ref	UK	C	+	+	C

The ribotypes of the *V. cholerae* strains examined, compared to those of selected reference strains of the El Tor and classical biotypes, are shown in Fig. 1. The ribotypes of different strains representing the three Matlab types of *V. cholerae*

were similar to the ribotypes of El Tor biotype strains and different from that of typical classical biotype strains described previously (11, 12). The ribotypes of two type I strains (lanes 9 and 10) were similar to that of toxigenic El Tor strains 1849 (lane 11), isolated in 2001, and G-3669 (lane 1) isolated in 1969 in Bangladesh. The Matlab type III strains belonged to three different ribotypes (Fig. 1, lanes 2 through 7), and the single type II strain had the same ribotype as a type III strain.

Classical and El Tor strains of *V. cholerae* are closely related but are not directly derived from each other (16, 17). El Tor vibrios appeared in Bangladesh, causing the first significant outbreak in 1968, and by 1973, they completely replaced the classical vibrios (1). In 1982, the classical biotype reappeared as the predominant epidemic strain in Bangladesh (25). In retrospect, it appears that classical cholera did not completely disappear from Bangladesh during the 1970s or late 1980s, but rather, its frequency varied in different regions of the country (26). The classical and El Tor biotypes have temporally overlapped over a decade and are likely to have interacted and exchanged genetic material either in the human intestinal milieu or in the aquatic environment. The strains isolated in this study probably represent an amalgam of such an exchange. It is well recognized that genetic exchange between divergent bacterial lineages can contribute importantly to the success of a species in complex and inconstant environments, such as those in which *V. cholerae* may reside. Several studies have also pointed to such exchanges as an important factor in *V. cholerae* population genetics and evolution (2, 3, 10).

On the basis of their phenotypic and genotypic traits, Matlab type I strains appeared to be more like the classical biotype while Matlab type II and III strains appeared to be more like the El Tor biotype. Matlab I strains, however, had altered phage receptor sites, since both of the strains were resistant to group IV and V phages. We assessed the similarity of the hybrid strains with classical and El Tor biotype strains on the basis of previously described ribotype patterns of classical and El Tor strains (11, 12). Ribotyping demonstrated that the Matlab I, II, and III strains showed minor differences in fragment patterns shown by the El Tor standard strains, suggesting that the hybrids originated from an El Tor-like clone. Therefore, overall, these strains were of the El Tor biotype displaying traits of the classical biotype. It

has been proposed that while El Tor and classical strains are not directly derived from each other but appear to be derived from environmental nontoxigenic strains that are El Tor-like (15). Clinical strains might become classical-like in some properties simply by loss of function, and this agrees with the findings disclosed herein. While some genetic exchange has also probably occurred, it appears that the strains have evolved classical biotype properties. With a *V. cholerae* genomic microarray that displayed more than 93% of the predicted genes of the whole genome sequence of El Tor strain N16961, Dziejman et al. (9) showed that only seven genes were absent solely in classical strains but present in other strains, leading them to speculate that classical biotype strains may be derived for a primordial environmental strain that was more El Tor-like than previously thought. Mitra et al. have previously reported the involvement of bacteriophage PS166 in the acquisition of some classical biotype-specific properties. By El Tor strains (22,23). Insertion of lysogenic phage genomes in the bacterial chromosome leading to the activation or inactivation of certain genes or expression of new phage-encoded genes is a natural phenomenon in the origination of genetic diversity. However, the present invention suggests that the acquisition of classical properties such as classical-type *tcpA* and *rstR* genes by El Tor vibrios by conversion through phage PS166 seems unlikely. It seems more probable that more than one genetic exchange was involved in the conversion of these strains. Irrespective of the mechanism involved in the generation of the natural hybrid strains, the existence of strains showing a combination of classical and El Tor biotype properties has evolutionary and epidemiological importance.

Interestingly, all of the hybrid strains carried the *tcpA* gene of the classical type. Recently, the dominance of the classical type *tcpA* gene among environmental strains of *V. cholerae* has been reported (6). The primary structure of TcpA is highly conserved among *V. cholerae* serogroups and biotypes shown to be pathogenic to humans, with amino acid identities of nearly 100% between strains of a given biotype and about 80% between classical and El Tor biotype O1 strains (20). It is not clear whether El Tor strains with classical *tcpA* are more efficient colonizers, but there is enough evidence showing that classical biotype strains elaborate abundant amounts of toxin-coregulated pilin when grown in vitro, in contrast to El Tor strains (20, 29).

The strains analyzed in the present study may well represent precursors of other clones that could lead to a pandemic spread since they have all of the genetic features needed to make a *V. cholerae* strain pandemic. Moreover, these strains were isolated from clinical cases of acute diarrhea. These strains also represent unique natural recombinants that could be judiciously employed in the construction of live-vaccine strains since they have a combination of virulence attributes of both the classical and El Tor biotypes of *V. cholerae* O1.

The classical biotype of *V. cholerae* O1 is believed to be extinct and has not been isolated for the past several years, even in southern Bangladesh, the last of the niches where this biotype prevailed. The data disclosed herein shows the existence of El Tor strains that have lost some of the El Tor phenotypes and acquired classical biotype characteristics. Therefore, even though strains that represent the classical biotype in entirety have been completely displaced, a reservoir of the virulence gene of the classical biotype still exists in nature. Previous molecular analyses of classical strains isolated between 1961 and 1992 in Bangladesh support the contention that classical vibrios were never completely replaced in Bangladesh (11). Thus, a vaccine developed against cholera must take this into consideration and must be targeted against both biotypes, failing which the global use of a vaccine exclusively against the El Tor biotype might select against El Tor strains and favor strains carrying the classical attributes, such as those isolated in this study.

These hybrid strains of *V. cholerae* may be more common than currently recognized because phenotypic methods are inadequate to precisely distinguish between the two biotypes and are not routinely used in clinical microbiology laboratories. IS1004 fingerprinting has determined that an O37 strain of *V. cholerae* that was responsible for a large outbreak of cholera in Sudan in 1968 (32) is closely related to classical O1 strains (4). This indicates that horizontal exchange of genes has occurred not only between O1 biotypes but also between classical biotype and non-O1 strains, and the Sudan strain is a typical example of how a novel genotype can cause a large outbreak. For these reasons, vaccines comprised of the strains of the invention should be particularly valuable in preventing such outbreaks.

**Example 3: Construction of non-toxicogenic *V. cholerae* strains**

The strains of *V. cholerae* serotype O1 described above were subjected to additional modifications to make them more suitable as vaccine strains, by removing the genes that encode cholera toxin, thus making them non-toxicogenic. Cholera toxin deleted derivatives of toxigenic *V. cholerae* strains were constructed as follows. Briefly, the method involves excision of the CTX prophage DNA which carries genes for cholera toxin. In toxigenic *V. cholerae*, chromosomal CTX prophage DNA is often flanked by copies of a related satellite phage genome RS1 which uses CTX $\phi$ -encoded proteins to form RS1 $\Phi$  particles. Different CTX-RS1 arrays exist in toxigenic *V. cholerae* strains.. We found that introduction of additional copies of RS1 element into toxigenic strains destabilized the chromosomal RS1-CTX array and led to excision of the integrated CTX prophage. The method consisted of superinfection of toxigenic strains with a genetically marked RS1 phage and passage of the strains in rabbit ileal loops followed by selection of strains which had lost the CTX phage as well as any unintegrated RS1 DNA.

**Strains, phages and plasmids.** Toxigenic *V. cholerae* strains used were isolated from the stools of cholera patients admitted to the Matlab hospital of the ICDDR,B. The genetically marked page DNA pRS1-Km was a derivative of the replicative form (RF) DNA of RS1 $\Phi$ , in which a kanamycin resistance (Kan<sup>R</sup>) determinant was introduced as described by Faruque et al. (33). The genetically marked RS1 satellite phage RS1-Km $\Phi$  was prepared from the culture supernatant of a control strain O395 transformed with pRS1-Km. This phage was used to infect recipient toxigenic *V. cholerae* strains by mixing defined quantity of bacteria and phage and incubating at 30°C. Transductants were selected by plating the mixture on culture plates containing kanamycin (50 $\mu$ g/ml).

Kan<sup>R</sup> colonies were picked and grown for several generations, and then tested for lack of CTX genes by using specific probes as described later. Representative colonies were also passaged in the ileal loops of rabbits and derivatives which had lost both CTX phage and pRS1-Km were identified as follows.

**Animal Experiments.** Selected colonies were grown in nutrient broth and passaged in ileal loops of adult New Zealand White rabbits obtained from the breeding facilities of ICDDR,B. Several short loops of approximately 6 to 8 cm in length were made in each rabbit which had previously been fasted for 48 hr. One ml of the cell suspension was inoculated into each loop by injection. After 18 hr., rabbits were sacrificed and the contents of the ileal loops were cultured on tarocholate-tellurite-gelatin agar (TTGA) plates. *Vibrio* colonies which became sensitive to kanamycin were identified and tested for the absence of CT genes by DNA hybridization and the presence of other relevant genes by PCR assays.

**Probes and PCR assays.** The gene probes used to detect the CTX phage genome were a 0.5 kb cloned fragment of the *ctxA* gene, an 840 bp region internal to the *zot* gene amplified by PCR, and a 2.1 kb *SphI-XbaI* fragment of pCTX-Km containing the entire *zot* and *ace* genes and part of *orfU*. Probes were labeled using a random primers DNA labeling kit (Invitrogen Corporation, Carlsbad, CA) and [ $\alpha$ - $^{32}$ P]ATP-deoxycytidine triphosphate (3,000 Ci/mmol, Amersham Biosciences, Uppsala, Sweden). Colony blots or Southern blots were prepared using nylon filters (Hybond, Amersham) and hybridized with the labeled probes following standard methods. PCR assays used in this study for different virulence associated genes included PCR assays specific for the *tcpA*, *tcpI* and *acfB* genes of the TCP pathogenicity island, and the *rstR* and *rstC* genes of the RS1-element. PCR reagents and kits were obtained either from Perkin-Elmer Corp. (Norwalk, CT) or Invitrogen Corporation and PCR was done essentially as described previously.

**ELISA for CT.** Strains were also tested for lack of CT production by the  $G_{M1}$ -ganglioside dependent enzyme linked immunosorbent assay ( $G_{M1}$ -ELISA). Using a rabbit anti-CT monoclonal antibody (Sigma Chemical Company, St. Louis, MO, USA). For each round of CT assay, 5 ml of AKI medium (1.5% Bactopeptone, 0.4% Yeast extract, 0.5 NaCl, 0.3%  $NaHCO_3$ , pH 7.4) was inoculated with approximately  $1 \times 10^3$  bacterial cells and grown for 16 hr at 30°C with shaking. The culture was centrifuged at 4000 X g for 5 min, and the supernatant was collected. Aliquots of the undiluted supernatant, 10 fold and 100 fold dilutions of the supernatant, and dilutions of purified CT (Sigma) were used for the toxin assay



following standard methods. Two strains were selected for further genetic manipulations and the attenuated strains were labeled as Matlab I and Matlab II. All required tests were done on these genetically manipulated strains to ensure that they do not produce cholera toxin and nor do they have the genes necessary for production of cholera toxin. These attenuated strains were also tested in animal models.

References and publications cited herein are listed below for convenience and are hereby incorporated by reference.

#### REFERENCES

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## WE CLAIM:

1. An isolated strain of *V. cholerae* having the identifying characteristics of a strain selected from the group consisting of Matlab I, Matlab II and Matlab III.
2. The strain of claim 1 wherein the identifying characteristics are phenotypic traits.
3. The strain of claim 1 wherein the identifying characteristics are genotypic traits.
4. The strain of claim 1 wherein the identifying characteristics are those of Matlab I.
5. The strain of claim 1 wherein the identifying characteristics are those of Matlab II.
6. The strain of claim 1 wherein the identifying characteristics are those of Matlab III.
7. An isolated *Vibrio cholerae* strain having the characteristics of Matlab I, II or III, deposited at the National Collection of Type Cultures, London, UK, on August 27, 2002 designated as NC13269-01, NC13270-01 or NC13271-01.
8. A biologically pure culture comprising *V. cholerae* having the identifying characteristics of a strain selected from the group consisting of Matlab I, Matlab II and Matlab III.
9. A vaccine for protection against cholera comprising *V. cholerae* having the identifying characteristics of *V. cholerae* selected from the group consisting of Matlab I, Matlab II and Matlab III.
10. The vaccine of claim 9 that is a killed whole cell vaccine.
11. The vaccine of claim 10 wherein the cells are killed by heat.
12. The vaccine of claim 10 wherein the cells are killed by formalin.
13. The vaccine of claim 9 that is an oral vaccine.
14. The vaccine of claim 9 wherein said *V. cholerae* is selected from the group consisting of *V. cholerae* as set forth in claim 7.
15. The vaccine of claim 9, wherein the number of organisms per dose of said *V. cholerae* is between about  $10^4$  and  $10^{16}$ .
16. The vaccine according to claim 9, wherein said *V. cholerae* is combined with at least one additional strain of *V. cholerae*.
17. The vaccine according to claim 9, wherein said *V. cholerae* is combined with a cholera toxoid.
18. The vaccine of claim 9, which is a combination vaccine.

19. The vaccine of claim 18, which includes vaccine components effective against at least one organism selected from the group consisting of rotavirus and enterotoxigenic *E. coli*.
20. The vaccine of claim 9, which is effective in humans.
21. A pharmaceutical composition comprising: *V. cholerae* having the identifying characteristics of *V. cholerae* Matlab I, II, or III and a pharmaceutically acceptable carrier.
22. The pharmaceutical composition according to claim 21, wherein said pharmaceutically acceptable carrier comprises sterile saline buffered from about pH 7.1 to about pH 7.3.
23. The pharmaceutical composition according to claim 21, wherein said pharmaceutically acceptable carrier is suitable for oral administration.
24. The pharmaceutical composition according to claim 21, wherein said *V. cholerae* is combined with at least one other strain of *V. cholerae*.
25. The pharmaceutical composition according to claim 21, wherein said *V. cholerae* is combined with a cholera toxoid.
26. The pharmaceutical composition according to claim 21 comprising a *V. cholerae* strain of claim 7.
27. A method of protecting humans against cholera comprising:  
obtaining a *V. cholerae* culture comprising a *V. cholerae* having substantially all of the identifying characteristics of *V. cholerae* selected from the group consisting of Matlab I, Matlab II, and Matlab III; and  
administering an effective amount of said culture to a human.
28. The method for protecting humans against cholera according to claim 27, wherein said culture is administered orally.
29. The method for protecting humans against cholera according to claim 27, wherein said effective amount is contained in a single dose.
30. Use of the strain of one of claims 1-7 in a vaccine or immunological composition.
31. An isolated strain of *V. cholerae* according to one of claims 1-7 that has been attenuated.

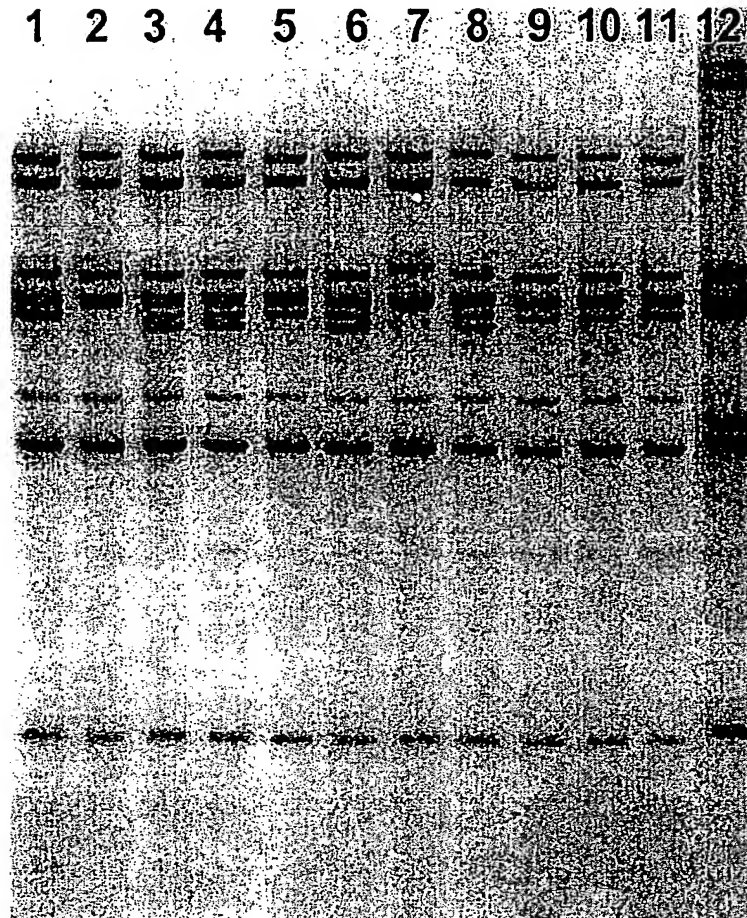
32. The isolated strain of claim 31 characterized in that the CTX prophage DNA that carries genes for cholera toxin has been excised.
33. The isolated strain of claim 31 that does substantially does not secrete cholera toxin.
34. The isolated strain of one of claims 31-33 that is designated \_\_\_\_\_ and deposited at the National Collection of Type cultures, London UK, on \_\_\_\_\_.
35. The use of the strain of one of claims 31-34 in a cholera vaccine or immunological composition.
36. A cholera vaccine or immunological composition comprising at least one of the strains of claims 31-34.
37. The vaccine of claim 36 that is a killed whole cell vaccine.
38. The vaccine of claim 37 wherein the cells are killed by heat.
39. The vaccine of claim 37 wherein the cells are killed by formalin.
40. The vaccine of claim 36 that is an oral vaccine.
41. The vaccine of claim 36 wherein said *V. cholerae* is selected from the group consisting of *V. cholerae* as set forth in claim 7.
42. The vaccine of claim 36, wherein the number of organisms per dose of said *V. cholerae* is between about  $10^4$  and  $10^{16}$ .
43. The vaccine according to claim 36, wherein said *V. cholerae* is combined with at least one additional strain of *V. cholerae*.
44. The vaccine according to claim 36, wherein said *V. cholerae* is combined with a cholera toxoid.
45. The vaccine of claim 36, which is a combination vaccine.
46. The vaccine of claim 45, which includes vaccine components effective against at least one organism selected from the group consisting of rotovirus and enterotoxigenic *E. coli*.
47. The vaccine of claim 36, which is effective in humans.

# ABSTRACT

The invention relates to a novel types of *Vibrio cholerae* that are useful for vaccines and immunological compositions.

10/526079

Figure 1





Rec'd PCT/PTO 28 FEB 2005

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/26968

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61 K 39/106; C12 N 1/21

US CL : 424/261.1, 235.1, 200.1; 435/243, 252.1, 252.3, 69.3, 909

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/261.1, 235.1, 200.1; 435/243, 252.1, 252.3, 69.3, 909

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Please See Continuation Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NAIR et al. New Variants of <i>Vibrio cholerae</i> O1 Biotype El Tor with Attributes of the Classical Biotype from Hospitalized Patients with Acute Diarrhea in Bangladesh. Journal of Clinical Microbiology, September 2002, Vol. 40, No. 9, Pages 3296-3299, whole document.	1-26 and 30-47
X	FARUQUE et al. Emergence of a new clone of toxigenic <i>Vibrio cholerae</i> O1 biotype El Tor displacing <i>V. cholerae</i> O139 Bengal in Bangladesh. J Clin Microbiol. March 1997, vol 35, No. 3, pages 624-630, Entire Document.	1-6, 8-26, 30-47
Y	US 5,653,986 A (MORRIS et al) Aug 5, 1997(05.06.1997) especially, column 2 and claim 4.	27-29



Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

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Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

Continuation of B. FIELDS SEARCHED Item 3:

WEST, MEDLINE, CAS ONLINE

search terms: Vibrio cholera, Matlab, Bangladesh, types, strains, isolated, diarrhea matlab, vaccine, attenuated, killed

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